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Photosynthesis, chloroplast ultrastructure, chemical composition and oxidative stress in *Theobroma cacao* hybrids with the lethal gene *Luteus-Pa* mutant

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Abstract

The *Theobroma* lethal character *Luteus-Pa* segregates in a 3:1 ratio, expresses in recessive homozygosis, initially inducing leaf chlorosis and finally provoking seedlings death. The objective of this work was to evaluate gas exchange, chlorophyll fluorescence emission, chemical composition and oxidative stress of wild and mutant seedlings resulting from the crosses Pa 30 × Pa 169 and its reciprocal, aiming to elucidate the seedlings death induced by *Luteus-Pa*. At 15 day after emergence (DAE) differences began to appear between the wild type and mutant. Mutant seedlings showed: (*1*) lack of photosynthesis and alterations in chloroplast morphology; (*2*) lower level of three abundant groups of proteins in leaves; (*3*) decrease in the content of chloroplastidic pigments (*4*) decrease in peroxidases activities and increase in leaf polyphenol oxidase activity; (*5*) decrease in carbohydrate and concentration of some nutrients and low dry mass in all plant parts. In leaves of mutant seedlings of both crosses damages occurred in the system responsible for the photochemical phase of photosynthesis. Variations in growth parameters and subsequent seedling death up to 60 DAE were related to exhaustion of cotyledonary reserves, inactive photosynthetic apparatus and oxidative stress.

Additional key words: cacao, lethal gene, mutants, Parinari, photosystem II.

Introduction

Cacao, *Theobroma cacao* L. (Malvaceae), is commercially explored for the production of seeds destined to the preparation of its most popular product, chocolate, and other derived and byproducts like cosmetics, fine beverages, jellies, ice creams, and juices. Cacao is an allogamous plant, with geographical origin in South America, with several series in the Amazon and Guyana regions (Almeida and Valle 2007).

Results on compatibility studies among genotypes of the Parinari (Pa) series of *T. cacao* resulting from the reciprocal crossing of Pa 30 × Pa 169 and Pa 121 × Pa 169 (Yamada *et al.* 1982), demonstrated the occurrence of the lethal simple recessive gene named *Luteus-Pa*. This character was identified for the first time in these series studying a physiological factor that expresses leaf chlorosis in seedlings of clones Pa 30, Pa 169, and Pa 121 and causes their death approximately in 30 to 40 d after germination (Almeida *et al.* 1998). These genotypes

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Abbreviations: C_i – intercellular CO_2 concentration; C_i/C_a – ratio between CO_2 in the intercellular mesophyll spaces and atmospheric CO_2; Chl(s) – chlorophyll(s); PAR – photosynthetically active radiation; CDM – cotiledonary dry mass; CV – coefficient of variation; DAE – day after emergence; DMSO – dimethyl sulfoxide; E – transpiration rate; F_o – minimum fluorescence of the dark-adapted state; F_m – maximum fluorescence of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximum potential quantum yield of PSII; G – starch grains; g_s – stomatal conductance to water vapour; ILA – individual leaf area; LDM – leaf dry mass; LN – leaf number; M – mucilage; Pa – Parinari; pI – isoeletric point; PODs – peroxidases; P_N – net photosynthetic rate; P_N/g_s – intrinsic water use efficiency; PPO – polyphenol oxidases; R_D – the dark respiration; RDM – root dry mass; R/S – root:shoot ratio; S – stroma; SHDM – shoot dry mass; SDM – stem dry mass; TDM – total dry mass; TLA – total leaf area; TSS – total soluble sugars.
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possess *Luteus-Pa* in heterozygosis and when crossing or selfing segregates in a 3:1 ratio (Bartley *et al.* 1983). This factor was used as a tool by Bartley (2005) and Yamada *et al.* (2005) to determine relations between genotypes belonging to that series. Physiological studies showed that the gene product acts in the photosystem II (PSII) reaction center (Almeida *et al.* 1998). The abnormalities are due to the action of one recessive allele, probably originating in specific individual mutations in the series, and are characterized mainly by causing lack of leaf pigmentation (Bartley 2005).

The population of the Pa series is constituted by a high number of promising genotypes that are used for genetic breeding of hybrid varieties resistant to *Phytophthora* sp. Therefore, due to the importance of the series for cacao breeding, we performed a detailed physiological study of recessive homozygous and wild type Pa seedlings aimed to understand and elucidate seedling death induced by the lethal factor *Luteus-Pa*.

Materials and methods

Plant material and growth conditions: The experiment was conducted in a greenhouse at the Universidade Estadual de Santa Cruz (UESC), Ilhéus, BA, Brazil (14°47'S, 39°16'W, 55 m a.s.l.) from April to July 2009. Micrometeorological parameters in the greenhouse were monitored through a climatological *Hobo Micro Station Data Logger* (*Onset*, USA) with internal sensors that record in *Windows* environment. Data collection and storage was automated with subsequent transfer *via* a serial port to a microcomputer. The maximum and minimum temperatures observed during the experimental period were 28 and 21°C, respectively. The minimum relative humidity recorded was 73% and the maximum 95%. The average value of PAR above the canopy was 194.6 μmol(photon) m^{-2} s^{-1}.

Seeds from Pa 30 × Pa 169 and Pa 169 × Pa 30 crosses, obtained by controlled pollination, were grown in polythene bags with 2.0-L capacity. The bags were filled with organic substrate (peat and shredded *Pinus* cortex + shredded coconut fiber in a 1:1 proportion), enriched with mineral macro- and micronutrients according to the nutritional needs of the species (Souza 2007).

Chlorophyll (Chl) fluorescence: Emission of Chl fluorescence was measured at 15 DAE in the second completely expanded and mature leaf, from the apex of the orthotropic axis, with a *Leaf Chamber Fluorometer* (*LI 6400-40*, Nebraska, USA), a LED-based fluorescence accessory for the portable photosynthesis system *LI-6400* (*LI-COR Bioscience, Inc*., Lincoln, Nebraska, USA). To assess the emission of Chl fluorescence in dark-adapted leaves, a clip was placed for 30 min in each leaf for solar radiation reflection, decrease leaf temperature, and oxidation of the whole photosynthetic electron transport system. The maximum quantum yield of PSII (F_V/F_m) was calculated by the equipment as [$F_V/F_m = (F_m - F_0)/F_m$)], where F_0 is the basal fluorescence yield measured after the illumination of a dark adapted sample with a measuring (modulated) light (0.25 kHz, < 0.1 μmol m^{-2} s^{-1}, 630 nm) and F_m is the maximum fluorescence yield of a dark-adapted sample obtained following a saturating light (modulated) pulse (20 kHz, 6,000 μmol m^{-2} s^{-1}, 630 nm, 0.8 s).

Leaf gas exchange: Leaf gas-exchange and fluorescence measurements were simultaneously performed with the *Li-6400*. Instantaneous measurements of leaf gas-exchange variables – net photosynthetic rate (P_N), stomatal conductance to water vapour (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) – were performed. In addition, P_N:photosynthetic active radiation (PAR) response curves were recorded between 08:00 and 12:00 h. The measurements were done at ten PAR levels [800, 600, 400, 200, 100, 50, 25, 10, 5, and 0 μmol(photon) m^{-2} s^{-1}]. The sequence was always initiated in decreasing order of PAR values. When instantaneous measurements were performed, the irradiance was set at 800 μmol m^{-2} s^{-1}, above the saturation irradiance of control seedlings.

The minimum pre-established time for reading stabilization, at each PAR level, was set at 60 s and the maximum, to save each reading, at 120 s. Also, the maximum admitted coefficient of variation (CV) to save each reading was 0.8%. During measurements, the leaf chamber (6 cm^2) temperature (26°C), the atmospheric CO_2 in the interior of the chamber (380 μmol mol^{-1}) and leaf-to-air water vapour-pressure deficit (1.0 ± 0.2 kPa) were kept constant. P_N, g_s, and E were estimated from variations in CO_2 and air humidity values in the interior of the chamber, as determined by the infrared gas analyzer of the device.

Parameters derived from light saturation data were obtained from the equation $P_N = P_{max}$ [1 – exp × (– α x PAR/P_{max})] – R_D, where P_{max} corresponds to the maximum rate of gross photosynthetic rate at saturation irradiance, α the apparent quantum efficiency of photosynthesis [μmol(CO_2) μmol^{-1}(photon)], and R_D the dark respiration (Iqbal *et al.* 1997). The ratio between CO_2 in the intercellular mesophyll spaces (C_i) and atmospheric CO_2 (C_a) (C_i/C_a) and the intrinsic water-use efficiency (P_N/g_s) were also estimated.

CO_2-response curves were measured under 800 μmol(photon) m^{-2} s^{-1} PAR, since it was previously found that this PAR value is higher than the photosynthetic light-saturation intensity for all measured leaves. Different CO_2 concentrations were obtained from 12-g CO_2 cartridges and automatically controlled by the

Li-COR 6400 CO_2 injector device (*6400-01 CO_2 injector*, *Li-cor Inc.* Lincoln, Nebraska, USA). The measurement sequence initiated at a 50 μmol mol^{-1} in chamber CO_2 concentration, increasing to 100, 200, 300, 500, 600, 700, 800, 900; 1,000; and 1,200 μmol mol^{-1} at 3–4 min intervals (Escalona *et al.* 1999). These rapid P_N/C_i curves were done to measure the CO_2-driven changes in P_N and not in g_s, whose response is slower. The values of P_N (CV < 1%) were stable 3–4 min after each in chamber CO_2 concentration change.

Growth parameters: Wild type and mutant seedlings of Pa 30 × Pa 169 and Pa 169 × Pa 30 crosses were harvested at the end of the experiment and divided in parts (root, stem, cotyledons, and leaves). After measurement of total (TLA) and individual (ILA) leaf area and determination of leaf number (LN) per plant, the parts were stored separately in paper bags and dried at 75°C to constant mass. After drying, root (RDM), shoot (SHDM), cotiledonary (CDM) and leaf (LDM) dry masses were determined. Leaf area was estimated with a *LI-3100* leaf area meter (*Li-cor Inc*., Lincoln, Nebraska, USA). Total dry mass (TDM) and the root:shoot ratio (R/S) were determined from the dry mass of the different vegetative organs.

Peroxidases and polyphenol oxidases activities: To analyze peroxidases (PODs; E.C.1.11.1.7) and polyphenol oxidases (PPOs; E.C. 1.10.3.1) activities, leaf samples of wild-type and mutant seedlings were collected at 15, 20, 25, and 30 DAE. The enzymatic extract and activity readings were carried out using a similar approach to the used by Pirovani *et al.* (2008). The conversion of the obtained data from absorbance values at 470 nm min^{-1} g^{-1}(DM) to guaiacol consumption in μmol h^{-1} g^{-1}(DM) can be done using the equation $y = 0.1284\ x + 0.0189$ ($R^2 = 0.99$), originated from a standard POD-guaiacol curve.

The readings of polyphenol oxidase activity were done at 395 nm and expressed in mmol(pirokathecol) g^{-1}(DM) h^{-1}, based on a straight line equation.

Proteins and Rubisco quantification: At 15 DAE Rubisco and total proteins content were determined. Total protein extract of leaves from wild and mutant seedlings was obtained, in triplicates, by phenolic extraction followed by precipitation with ammonium acetate at 0.1 M in methanol (Pirovani *et al.* 2008). Proteins were quantified using the 2D *Quanti Kit*, according to the manufacturer recommendations (*GE Healthcare*, UK). Samples containing 30–50 μg of leaf proteins from both types of seedlings were analyzed in SDS-PAGE at 12.5% (Laemmli 1970). For 2D (SDS-PAGE), visualization and quantification of data sets was performed according to methodology described by Pirovani *et al.* (2008).

Mass spectrometry analysis: For protein sequencing, the spots were excised from the 2D gel, washed with a solution of 25 mM NH_4HCO_3 and 50% acetonitrile at pH 8.0. The extracted proteins were digested in a trypsin solution at 37°C for 16 h. Afterwards, the peptides extraction was done with a solution of 50% acetonitrile and 5% formic acid (Yin *et al.* 2005). The solution containing the proteolytic digests was first fractionated in a ionic exchange column. Subsequently, the digests were fractionated on a reversed-phase C18 column, using two mobile phases, phase A containing H_2O and 0.1% formic acid and phase B with acetonitrile and 0.1% formic acid. For peptides separation, a linear gradient of 5–95% acetonitrile was used. The eluted peptides were directly introduced to the quadrupole mass spectrometer *Q-TOFmicro* (LC-MS/MS*, Waters*, Milford, MA) by its ele**c**trospray probe. The most abundant ions, observed in the MS spectrum were automatically selected for collision-induced dissociation (CID) using the software *Masslynx*, generating their MS/MS spectra. Argon was used for peptides collision.

The resulting spectra were processed by the MaxEnt3 algorithm of the *Masslynx ProteinLynx* software to generate a list of masses, which correspond to peaks obtained in the spectra analyzed. The list of peaks generated by *Proteinlynx 2.3* was searched against databases of *T. cacao* genome, NCBI and SWISSPROT. For this search we used version *2.1.0* of *MASCOT* (*Matrix Science*, Portland, OR). The identification was made by Peptide Mass Fingerprint and sequenced by MS/MS.

Mineral macro- and micronutrients determination: In roots, stems, and cotyledons, and leave's dry masses the contents of some macro- and micronutrients were determined. After nitro-perchloric digestion, concentrations of Ca, Mg, Fe, Zn, Cu and Mn were determined by atomic absorption spectrophotometry, using atomic absorption spectrophotometer *CG7000-SBC* (*Perkin Elmer*, Massachusetts, USA). Concentrations of P by colorimetry utilizing the vitamin C approach (Braga and Defelipo 1974) using spectrophotometer model *B572-A* (*Micronal*, Ludwigshafen, Germany) and K by flame emission photometry with flame photometer *B462* (*Micronal*) (Isaac and Kerber 1971). Nitrogen was determined by Kjeldahl after sulphosalicylic digestion of the samples (Jackson 1958), using equipment micro Kjeldahl TE0363 (*Tecnal*, Piracicaba, SP).

Total soluble sugars and starch: In all plant parts, concentration of total soluble sugars (TSS) was determined by the anthrone method (Clegg 1956). Starch concentration was estimated by the method described by McCready *et al* (1950).

Photosynthetic pigments: The concentration of chloroplast pigments was determined in leaves of all seedlings by the methodology described by Hiscox and

Israelstam (1979), with some modifications. After incubation of three leaf disks (0.84 cm^2) with 4 mL of dimethyl sulfoxide (DMSO) saturated with $CaCO_3$ at 65ºC for 12 h (Wellburn 1994), the absorbance of the extracts were read at λ of 665, 649, and 480 nm in a *Hitachi U-2000* double-beam spectrophotometer (*Hitachi Instruments Inc*., Danbury, CA, USA) to determine Chls *a* and *b* and carotenoids, respectively. Total Chl content was calculated by Wellburn (1994) equations for DMSO extracts.

Transmission electron microscopy (TEM) analyses were carried out at the UESC Electron Microscopy Centre to evaluate chloroplasts in mature leaves of wild type and mutant seedlings. TEM slides were stained with uranyl acetate and lead citrate. Leaf tissues were immersed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9), cut into small fragments (*ca.* 1 mm^3), submitted to a weak vacuum for 30 min and subsequently maintained under normal pressure for a further 1 h. Samples were then submitted to six washes (10 min each) with sodium cacodylate buffer, fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 4 h at 4ºC, washed six times (10 min each) with sodium cacodylate buffer, and dehydrated in an ethanol gradient (30, 50, 75, 85, and 95% ethanol, followed by three washes in 100% ethanol). Finally, samples were covered sequentially with solutions of propylene oxide: epoxy resin (Spurr 1969) in proportions of 3:1, 1:1, and 1:3, and then three times with pure Spurr resin, the final treatment being continued overnight at room temperature. Next day, samples were placed in silicon moulds, covered with pure Spurr resin and polymerized overnight. The polymerized resin blocks were trimmed with a razor blade, thinly sectioned (2 µm) with a glass blade, and ultrathinly sectioned (50–60 nm) with a diamond blade using a microtome. Thinly-cut sections were placed between glass slides and cover slips for structural examination. Ultrathin sections were cut and placed onto copper mesh grids and examined using a *Morgagni™ 268D TEM* (*FEI Company*, Hillsboro, OR, USA) equipped with a CCD camera and controlled by software running under *Windows OS*.

Statistical analysis: The data set was analyzed as a complete randomized design with four treatments, referring to the two crosses and the two progenies (wild type and mutants), five replications for chemical composition and biomass data, 12 replications for leaf gas exchange and Chl fluorescence emission data, and one seedling per experimental unit. Statistical differences were detected by *t*-test and *Tukey* test ($p<0.01$, $p<0.05$). Linear regressions for the evaluated physiological parameters were also done.

Results

The expression of the lethal gene *Luteus-Pa* at 15 DAE was found in a quarter of the resultant progeny from the crosses Pa 30 × Pa 169 and Pa 169 × Pa 30 showing segregation in a 3:1 proportion. Leaves of seedlings bearing the lethal allele showed chlorosis and, after exhaustion of the cotiledonary reserves, necrotic spots, followed by death at 60 DAE (Fig. 1). Therefore, the progenies did not differ in the segregation pattern among themselves (Fig. 2), which shows that the inheritance of that gene is nuclear, simple, and no maternal.

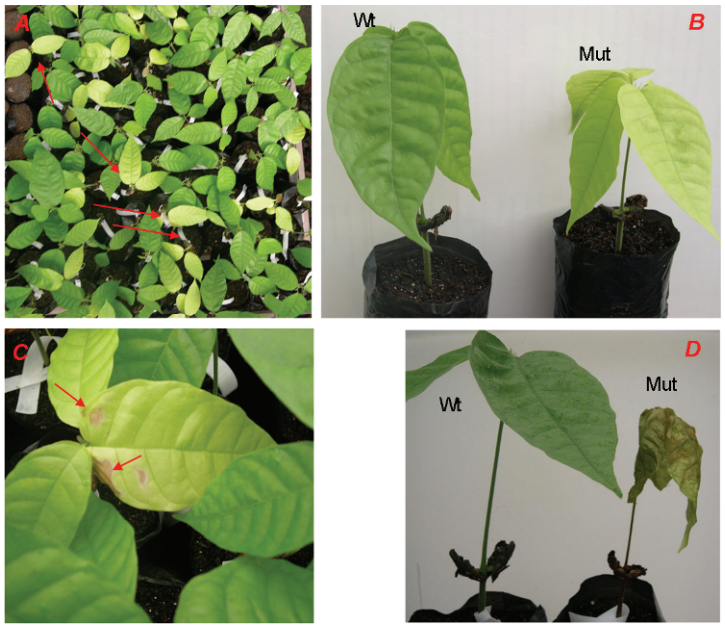

Fig. 1. Mutant and wild-type seedlings of the hybrid Pa 169 × Pa 30 at 15 (*A*, *B*), 35 (*C*) and 45 (*D*) days after emergence. Note the leaf chlorosis in the mutants seedlings (*A, B, C, red arrows*), followed by necrotic points in the leaf (*C*, *red arrows*) and seedling death (*D*).

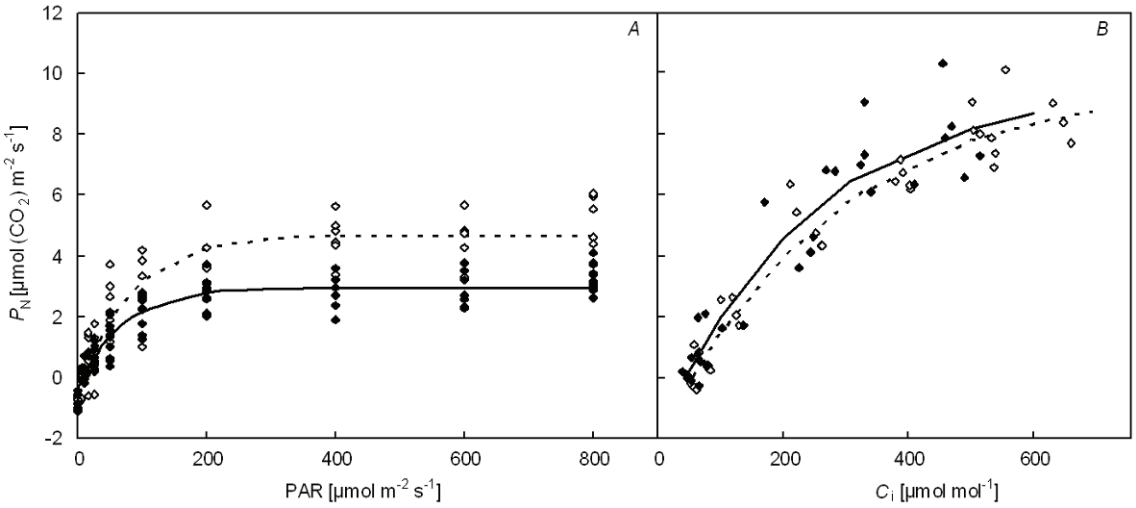

Fig. 2. Response curve of leaf net photosynthesis (P_N) as a function of photosynthetically active radiation (PAR) (*A*) and CO_2 intercellular concentrations (C_i) (*B*) in *Theobroma cacao* seedlings of the wild type resulting from the crosses Pa 30 × Pa 169 (♦, *solid line*) and Pa 169 × Pa 30 (◊, *dotted line*) at 15 days after emergence.

Table 1. Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration (E), intrinsic water-use efficiency (P_N/g_s), ratio between internal (C_i) and atmospheric (C_a) CO_2 (C_i/C_a), and dark respiration (R_D) of wild-type and mutant *Theobroma cacao* seedlings at 15 days after emergence. Means of 12 replications ± SE. R_D estimated at PAR = 0, all other variables at PAR> 500 µmol(photon) m^{-2} s^{-1}. Means comparison were done using *t* test. * $p<0.05$; ** $p<0.01$.

Variable	Pa 30 × Pa 169 Mutant	Wild type	Pa 169 × Pa 30 Mutant	Wild type
P_N [µmol(CO_2) m^{-2} s^{-1}]	–0.31 ± 0.150**	4.83 ± 0.270	–0.66 ± 0.09**	3.08 ± 0.12
g_s [mol(H_2O) m^{-2} s^{-1}]	0.01 ± 0.001**	0.03 ± 0.002	0.01 ± 0.002**	0.03 ± 0.002
E [mmol(H_2O) m^{-2} s^{-1}]	0.21 ± 0.020**	0.56 ± 0.050	0.16 ± 0.03**	0.52 ± 0.04
P_N/g_s [µmol(CO_2) $mol^{-1}(H_2O)$]	–29 ± 12**	157 ± 8.000	–65 ± 26**	120 ± 9.0
C_i/C_a	1.09 ± 0.050**	0.35 ± 0.030	1.24 ± 0.11**	0.46 ± 0.04
R_D [µmol(CO_2) m^{-2} s^{-1}]	–0.77 ± 0.260**	–0.80 ± 0.110	–0.75 ±0.11*	–0.72 ±0.14

Gas exchange and Chl emission: At 15 DAE a significant difference in gas-exchange parameters was observed between wild-type and mutant seedlings. Average values of P_N, g_s, E, and P_N/g_s shown by mutant seedlings leaves were lower than the wild type values (Table 1). Actually, P_N and P_N/g_s values were negative indicating that the mutant seedlings were just respiring. Furthermore, C_i/C_a values were higher in the *Luteus-Pa* bearing seedlings than in the wild type seedlings (Table 1).

P_N:C_i curves showed that there were no responses of the photosynthetic apparatus to the increase in internal CO_2 in the mutant seedlings of both crosses when compared to wild-type seedlings (Figs. 3*A*,*B*). Similar response was also found in regard to P_N:PAR curves in which P_N values of mutant seedlings approached zero. In contrast, the wild-type seedlings showed values for P_N, g_s, and E of 4.83 µmol(CO_2) m^{-2} s^{-1}, 0.03 mol(H_2O) m^{-2} s^{-1} and 0.56 mmol(H_2O) m^{-2} s^{-1}, respectively (Figs. 3*C*,*D*).

The measurements of Chl fluorescence emission at 15 DAE show higher F_0 values for the mutants than for wild-type seedlings while F_m was higher in the wild type (Table 2). The obtained nearly-zero values of F_v/F_m parameter documented absence of PSII activity in the mutant seedlings.

Dry matter: Evaluation of growth parameters at 15 DAE showed a tendency in CDM and RDM of mutant seedlings to decrease. Also, a significant reduction ($p<0.05$) in LDM, SHDM, and TDM values of mutant seedlings of both crosses was found. TLA, ILA, R/S ratio, and LN values did not statistically differ ($p<0.05$) from those of the wild-type seedlings (Table 3).

Enzymes: The activity of peroxidases was significantly ($p<0.05$) higher in the mutant leaves than that measured in leaves of wild type seedlings (except at 20 DAE) (Fig. 4). Since no significant differences were found in peroxidases activity between progenies of both crosses at 15 DAE, activity of these enzymes were determined only in one cross (Pa 169 × Pa 30) at 20, 25, and 30 DAE (Fig. 5).

As regards polyphenol oxidases, it was found that

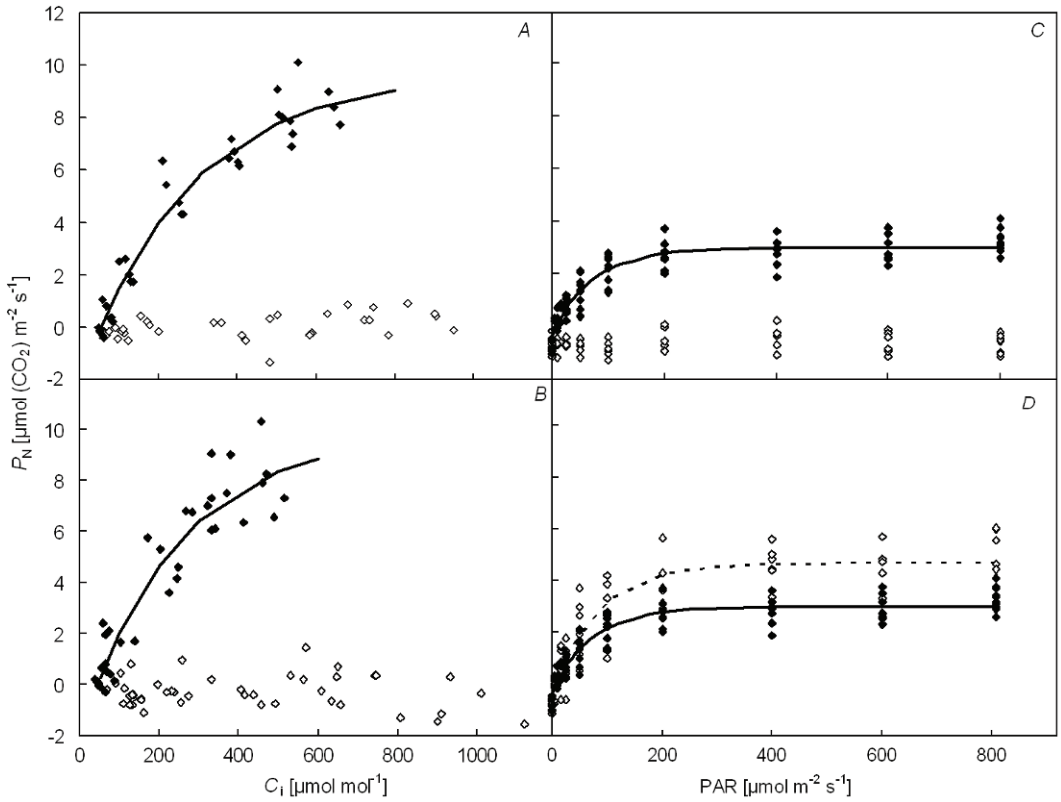

Fig. 3. Response curve of leaf net photosynthesis (P_N) as a function of CO_2 intercellular concentration (C_i) (*A, B*) and photosynthetically active radiation (PAR) (*C, D*) in wild-type (♦) and mutant (◊) seedlings of *Theobroma cacao* resulting from the crosses Pa 30 × Pa 169 (*A, C*) and Pa 169 × Pa 30 (*B, D*) at 15 days after emergence.

Table 2. Variables of leaf chlorophyll fluorescence emission of wild-type and mutant *Theobroma cacao* seedlings at 15 days after emergence. Means of 12 replications ± SE. Means comparison were done using *t*-test. $^{**}p<0.01$.

Crosses	Treatment	F_o	F_m	F_v/F_m
Pa 30 × Pa 169 [H_1]	Mutant	$630 \pm 67^{**}$	$634 \pm 67^{**}$	$0.008 \pm 0.0014^{**}$
	Wild type	357 ± 6	1441 ± 201	0.752 ± 0.0054
Pa 169 × Pa 30 [H_2]	Mutant	$984 \pm 58^{**}$	$983 \pm 59^{**}$	$0.0003 \pm 0.0002^{**}$
	Wild type	388 ± 12	1431 ± 11	0.7289 ± 0.0090

the activity values of these enzymes were significantly ($p<0.05$) lower in leaves of mutant than in leaves of wild type seedlings (Fig. 5).

Proteins: The large and small (L and S, respectively) subunits of Rubisco (EC 4.1.1.39) were identified in both mutant and wild type seedlings leaves. The values were not significantly different from each other (Fig. 6). At 15 DAE the electrophoretic profile of total proteins in 2D in seedlings bearing *Luteus-Pa* were characterized by showing a group of proteins at higher concentrations (pH = 4.7–8.0, 46 kDa; pH= 5.0–6.0, 20.1 kDa) and three in smaller concentration (pH = 5.0, 30 kDa; pH = 6.0, 30 kDa; pH = 8.0, 23 kDa) in their leaves, normalized data in relations to global means of the spots intensity (Fig. 7). The results of sequencing allowed the identification of four proteins: (*1*) larger subunits of Rubisco (group 1 and 3); (*2*) PsbO (group 2), a protein essential for PSII activity, present in lower concentrations in the mutant compared with the wild-type genotype (four times smaller) and (*3*) a protein trypsin inhibitor (group 4). The presence of the lethal gene may have interfered in the synthesis of PsbO, producing a defective protein, and thus blocking the photosynthetic electron transport chain (Table 7). Total protein concentration did not differ statistically between both types of seedlings (Fig. 8).

Mineral nutrients: In general, there were significant differences ($p<0.05$) in the concentration of macro and micronutrients in the vegetative organs of the wild and mutant seedlings (Table 4). Wild type seedlings showed significantly higher ($p<0.05$) concentrations of Zn and

Table 3. Growth parameter of wild-type and mutant seedling of *Theobroma cacao* resulting from the reciprocal cross between Pa 169 and Pa 30 at 15 days after emergence. Means of 12 replications ± SE. Means comparison were done using *t*-test. * $p<0.05$; ** $p<0.01$. Root (RDM), stem (SDM), cotyledon (CDM), leaf (LDM), shoot (SHDM), and total dry mass (TDM), root to shoot dry mass ratio (R/S); total (TLA) and individual leaf area (ILA); leaf number (LN). NS = not significant.

Growth variables	Treatment	
	Wild type	Mutant
RDM [g]	0.20 ± 0.01^{NS}	0.16 ± 0.02
SDM [g]	0.28 ± 0.03^{NS}	0.28 ± 0.04
CDM [g]	0.45 ± 0.03^{NS}	0.38 ± 0.04
LDM [g]	$0.45 \pm 0.02^{**}$	0.28 ± 0.03
SHDM [g]	$1.16 \pm 0.06^{*}$	0.94 ± 0.07
TDM [g]	$1.38 \pm 0.07^{*}$	1.10 ± 0.08
R/S	0.15 ± 0.01^{NS}	0.15 ± 0.02
TLA [m^2]	0.02 ± 0.01^{NS}	0.01 ± 0.01
ILA [m^2 $plant^{-1}$]	0.004 ± 0.01^{NS}	0.003 ± 0.01
LN	4.17 ± 0.17^{NS}	4.17 ± 0.17

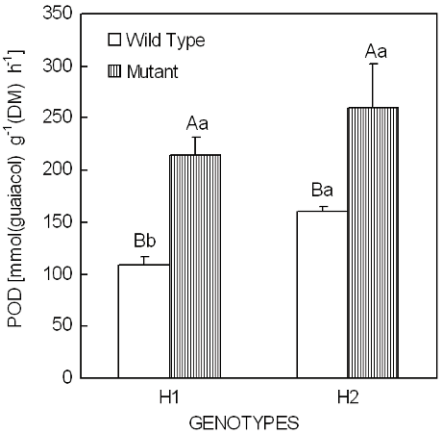

Fig. 4. Peroxidases (POD) activity in leaves of wild type and mutant seedlings of *Theobroma cacao* resulting from the crosses Pa 30 × Pa 169 (H1) and Pa 169 × Pa 30 (H2) at 15 days after emergence. (┬) − mean standard error. Number of replicates (n = 5). *Lowercase letters* indicate comparisons between sampling times, *uppercase letters* comparisons between wild type and mutant seedlings. Mean comparisons were made using *Tukey* test ($p<0.05$).

Mn in root and leaves, Fe in the cotyledons and statistically lower K concentratration in the stem and N, P, Ca, and Mg in the leaf when compared to mutant seedlings. In all vegetative organs of the mutants Fe concentration showed a decreasing tendency and Zn significant decreases ($p<0.05$) in roots and leaves (Table 4).

Carbohydrates concentration did not differ statistically between both types of seedlings at 15 DAE (Table 5) except for starch in the stems. At that time, the higher TSS concentrations values were found in cotyledons, while starch values were higher in stems of both wild and mutant seedlings.

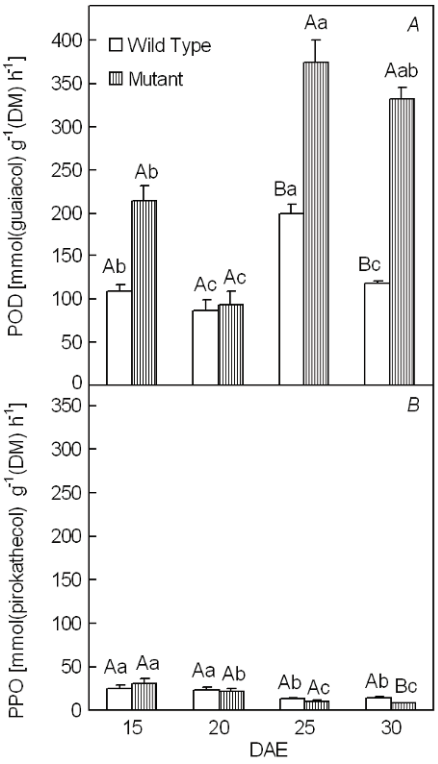

Fig. 5. Peroxidases (POD; *A*) and polyphenol oxidases (PPO; *B*) activities in leaves of wild-type and mutant seedlings of *Theobroma cacao* resulting from the cross Pa 169 × Pa 30 at 15, 20, 25, and 30 days after emergence. (┬) – mean standard error. Number of replicates (n = 5). *Lowercase letters* indicate comparisons between sampling times, *uppercase letters* comparisons between wild type and mutant seedlings. Mean comparisons were made using *Tukey* test ($p<0.05$).

Chl concentration: Mutant seedlings showed lower concentrations of Chls *a* and *b* and carotenoids and lower index of pheophytination than wild seedlings, with decreases of 48, 34, 42, and 0.85%, respectively, at 15 DAE, period in which measurements of leaf gas exchanges were made (Table 6). At 60 DAE, when the degradation of chloroplastidic pigments and the exhaustion of the cotyledonary reserves were complete, the mutant seedlings presented necrotic leaf spots typical of apoptosis, dying shortly after.

TEM analysis: The transmission electron microscope reveals finer details of chloroplasts in the mesophyll cells of the fully expanded green leaves in wild type seedlings showing the membranes of the chloroplast envelope, grana and stroma (S) lamellae containing stacks of thylakoids, mucilage (M) and starch grains (G) (Fig. 9*A*). In contrast, chloroplasts in mutant seedlings exhibited significant structural changes, specially the stroma lamellar system was disorganized and contained invaginations, forming spaces between the thylakoids membranes. Large and small chloroplasts can be also observed in the mutant seedlings (Fig. 9*B*).

Discussion

Expression of the lethal gene *Luteus-P*a at 15 DAE (Fig. 1) confirmed the 0.25 death rate verified in previous works carried out with members of the Parinari series by Yamada *et al.* (1982), Bartley *et al.* (1983), and Yamada *et al.* (2005). Additionally, the 3:1 phenotypic proportions of wild type to mutant plants affected by chlorosis in both progenies of reciprocal crosses confirm that the gene that determines this character is located at the nucleus and not in mitochondria or chloroplast. Some seedlings of the crosses carried out by Almeida *et al.* (1998) between genotypes of the Pa series also showed leaf chlorosis in the whole leaf, followed by necrotic spots and, afterwards, seedling death at 60 DAE. According to these authors, the occurrence of necrosis was due to the decrease in photosynthetic pigments and exhaustion of the cotyledonary reserves. For Bartley (2005), those abnormalities can be caused by the action of a simple recessive allele arising from specific individual mutations in that series or in some genotypes that are characterized for absence of leaf pigmentation.

Gas exchange and fluorescence: The negative P_N values of mutants seedlings (Fig. 3) can be related to injuries in the reaction center of PSII (Almeida *et al.* 1998), causing high C_i, decrease in g_s, and low Chl fluorescence emission (Logan *et al.* 2007) (Tables 1, 2). This is because P_N depends on the CO_2 assimilation pathways that determine the potential assimilation of carbon (Paul and Pellny 2003). It is considered that the relation C_i/C_a is an appropriate indicator for the limitations of photosynthesis due to stomata (Farquhar and Sharkey 1982). This was corroborated by the fact that the C_i/C_a values of mutant seedlings were higher when compared to values of the wild type (Table 1). Mutant seedlings showed negligible F_V/F_m values in comparison with wild seedlings (Table 2). This was accompanied by increased level of F_o indicating an increased fluorescence emission by antenna pigments that cannot transfer absorbed energy to PSII reaction center, most probably due to its damage.

Biomass: The accentuated decrease in cotyledonary biomass of mutant seedlings showed that these organs are the only reserve source for growth maintenance in these seedlings (Table 3). Respiration and CO_2 assimilation determine dry mass production since seedling growth depends on photosynthesis (Hollinger 1987). Biomass is the main determinant for respiration and the leaf area for photosynthesis (Hollinger 1987).

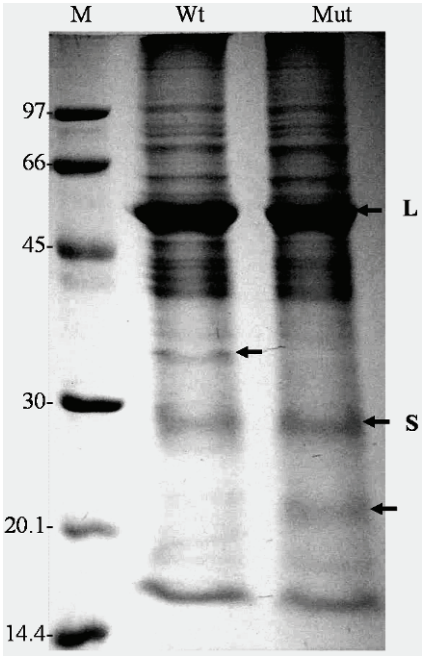

Fig. 6. Identification of the large (L) and small (S) subunits of Rubisco in leaves of wild-type (Wt) and mutant (Mut) seedlings of *Theobroma cacao* resulting from the cross Pa 169 × Pa 30 at 15 days after emergence (DAE). Analyses in SDS-PAGE 12.5 % at 15 DAE. Gel coloured with Coomassie blue. M – molecular mass standard. *Arrow* indicates presence of the selected abundant proteins.

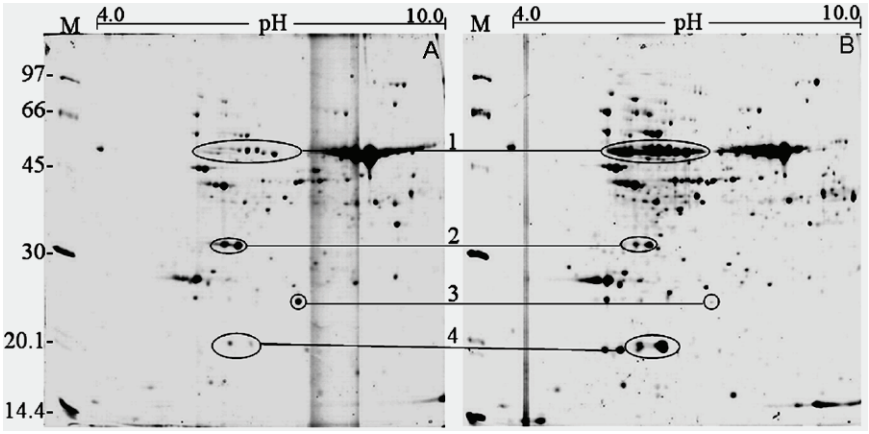

Fig. 7. Electrophoretic 2D profile of proteins from leaves of wild (*A*) and mutant (*B*) *Theobroma cacao* seedlings resulting from the cross Pa 169 × Pa 30. M – molecular mass standard.

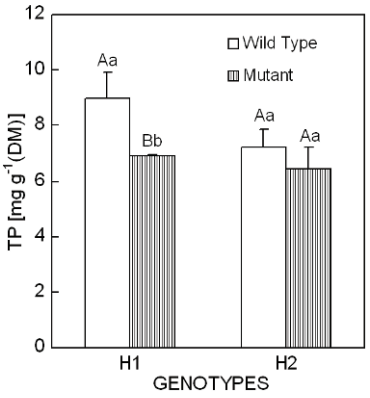

Fig. 8. Total protein concentration (TP) in leaves of wild and mutant seedlings of *Theobroma cacao* resulting from the crosses Pa 30 × Pa 169 (H1) and Pa 169 × Pa 30 (H2) at 15 days after emergence. (┬) – mean standard error. *Lowercase letters* indicate comparisons between sampling times, *uppercase letters* comparisons between wild type and mutant seedlings. Mean comparisons were made using *Tukey* test (p<0.05).

Mineral nutrients: The decrease of Fe content in plants is associated to decreases in the concentration of Chls and others photosynthetic pigments like xanthophylls and carotenes (Welch 1995). It is also associated to the activity of electron transporters of PSII, resulting in reduction of photosynthesis. The decline in Zn concentrations found in the mutant seedlings was due to inhibition of photosynthesis by the *Luteus-Pa* gene. It also suggests that zinc deficiency impairs the biochemical capacity of the plant to fix CO_2 (Randall and Bouma 1973). This would be in agreement with the results of Spencer and Possingham (1960), who established a depressed Hill reaction activity in chloroplasts isolated from zinc-deficient spinach plants, and with the demonstration that zinc-deficient soybeans contain reduced levels of Rubisco (Jyung *et al.* 1972). Low concentrations of these two nutrients show characteristic symptoms like chlorosis and subsequent leaf necrosis (Welch 1995), as shown by the seedlings bearing the lethal factor.

Table 4. Macro- and micronutrient concentrations in wild-type and mutant seedling of *Theobroma cacao* resulting from the reciprocal cross between Pa 169 and Pa 30 at 15 days after emergence. Means of 5 replications ± SE. *Uppercase letters* compare treatments and *lowercase* ones within treatments. Mean comparisons were made using *Tukey* test (p<0.05).

Treatment	Organ	Macronutrients N [g kg^{-1}(DM)]	P [g kg^{-1}(DM)]	K [g kg^{-1}(DM)]	Ca [g kg^{-1}(DM)]	Mg [g kg^{-1}(DM)]
Wild type	Root	11 ± 2.0^{Ac}	2.18 ± 0.28^{Ab}	11 ± 0.3^{Ab}	1.47 ± 0.25^{Ac}	1.71 ± 0.22^{Ac}
	Stem	14 ± 3.0^{Abc}	4.36 ± 0.55^{Aa}	12 ± 0.5^{Bb}	4.02 ± 0.24^{Ab}	5.48 ± 0.32^{Ab}
	Cotyledon	18 ± 0.6^{Aab}	4.19 ± 0.09^{Aa}	12 ± 3.7^{Ab}	6.44 ± 0.37^{Aa}	6.67 ± 0.19^{Aa}
	Leaf	24 ± 0.2^{Ba}	4.52 ± 0.12^{Ba}	41 ± 1.0^{Aa}	5.53 ± 0.13^{Ba}	6.72 ± 0.10^{Ba}
Mutant	Root	10 ± 0.2^{Ac}	1.86 ± 0.16^{Aa}	10 ± 0.8^{Ac}	1.34 ± 0.12^{Ac}	1.34 ± 0.19^{Ac}
	Stem	11 ± 0.4^{Ac}	6.16 ± 0.39^{Aa}	33 ± 0.9^{Ab}	3.09 ± 0.57^{Ab}	4.11 ± 0.90^{Ab}
	Cotyledon	19 ± 0.8^{Ab}	8.04 ± 0.30^{Aa}	16 ± 0.6^{Abc}	5.94 ± 0.31^{Aa}	6.75 ± 0.25^{Aa}
	Leaf	35 ± 1.5^{Aa}	6.53 ± 0.49^{Aa}	57 ± 5.3^{Aa}	6.98 ± 0.34^{Aa}	8.31 ± 0.52^{Aa}

Treatment	Organ	Micronutrients Fe [mg kg^{-1}(DM)]	Zn [mg kg^{-1}(DM)]	Cu [mg kg^{-1}(DM)]	Mn [mg kg^{-1}(DM)]
Wild type	Root	420 ± 15.4^{Aa}	25 ± 1.9^{Ad}	9.5 ± 0.9^{Ac}	162 ± 10^{Ac}
	Stem	192 ± 10.2^{Aa}	49 ± 0.8^{Ac}	9.5 ± 0.6^{Ac}	242 ± 10^{Ab}
	Cotyledon	369 ± 10.0^{Aa}	60 ± 2.5^{Ab}	12.5 ± 0.6^{Ab}	332 ± 15^{Ab}
	Leaf	188 ± 11.0^{Aa}	98 ± 1.0^{Aa}	16.0 ± 0.7^{Aa}	464 ± 14^{Aa}
Mutant	Root	342 ± 7.20^{Aa}	16 ± 0.9^{Bd}	9.5 ± 0.9^{Ac}	113 ± 2.0^{Ba}
	Stem	157 ± 9.03^{Aa}	39 ± 0.9^{Ac}	12.2 ± 0.7^{Abc}	311 ± 1.0^{Abc}
	Cotyledon	267 ± 9.38^{Bb}	63 ± 0.4^{Ab}	$14.5 \pm 1{,}0^{Aab}$	379 ± 1.5^{Ab}
	Leaf	165 ± 6.75^{Aa}	74 ± 3.4^{Ba}	17.6 ± 0.9^{Aa}	400 ± 1.5^{Bc}

Peroxidases and polyphenol oxidases: Plant peroxidases activity is expected to increase under some kind of stress (Gaspar *et al.* 1985, Goldberg *et al.* 1986). The peroxidases activity demonstrates a close relationship with the changes in physiological processes as respiration, photosynthesis, and transpiration, and show potentialities to act as a sensitivity indicator of the committed metabolic activity (MacFarlane and Burchett 2001). In the present work, the higher activity of peroxidases in the mutant seedlings (Figs. 4, 5) was probably due to damages in PSII provoked by the expression of the lethal character *Luteus-Pa*.

Most polyphenol oxidase activity occurs in chloroplasts. Its occurrence in plant cells depends on the developmental stage, species and age, while its activity depends on light (Dogan *et al.* 2005). Mutant plants were characterized as more tender and showing a deficient photosynthetic apparatus regarding light absorption when compared with wild type seedlings. This justifies in part the low PPO activity in those seedlings. Besides, it may indicate that there was low lignification of the cell wall of the mutants compared to the wild type seedlings at the four sampling times (15, 20, 25, and 30 DAE), since PPO is related to lignification (Alba *et al.* 2000).

Table 5. Starch and total soluble sugars (TSS) concentrations in wild-type and mutant seedlings of *Theobroma cacao* resulting from the cross Pa 169 × Pa 30, at 15 days after emergence. Means of 5 replications ± SE. Mean comparisons were made using *Tukey* test ($p<0.01$). *Uppercase letters* compare treatments and *lowercase* ones within treatments.

Treatments	Organ	Carbohydrates Starch [mg g^{-1}(DM)]	TSS [mg g^{-1}(DM)]
Wild type	Root	19 ± 3.4^{Ab}	30 ± 1.0^{Ab}
	Stem	103 ± 12.7^{Aa}	30 ± 9.40^{Ab}
	Cotyledon	49 ± 6.3^{Ab}	109 ± 2.4^{Aa}
	Leaf	27 ± 1.6^{Ab}	39 ± 2.0^{Ab}
Mutant	Root	12 ± 2.3^{Ab}	27 ± 0.5^{Ab}
	Stem	45 ± 3.5^{Ba}	28 ± 3.6^{Ab}
	Cotyledon	34 ± 4.6^{Aa}	104 ± 10.3^{Aa}
	Leaf	26 ± 3.7^{Aab}	24 ± 3.1^{Ab}

Table 6. Concentration of chlorophyll *a* (Chl *a*), *b* (Chl *b*) and total (Chl t), carotenoids (C) and pheophytin index (IP) in wild-type and mutant *Theobroma cacao* seedlings from the cross Pa 169 × Pa 30 at 15 days after emergence. Means of 5 replications ± SE. Mean comparisons were made using *t*-test. ** $p<0.01$.

Treatment	Pigments Chl *a* [mg dm^{-2}]	Chl *b* [mg dm^{-2}]	Chl t [mg dm^{-2}]	C [mg dm^{-2}]	IP [mg dm^{-2}]
Wild type	$26.8 \pm 0.15^{**}$	$11.0 \pm 0.06^{**}$	$37.8 \pm 0.19^{**}$	$5.34 \pm 0.06^{**}$	$4.70 \pm 0.01^{**}$
Mutant	13.5 ± 0.11	7.27 ± 0.09	20.8 ± 0.19	3.11 ± 0.0	4.66 ± 0.01

Table 7. The proteins identified by mass spectrometry (*see* Fig. 7). MM – molecular mass.

Group No.	Protein Name	Theoretical pI	Theoretical MM [kDa]	Sequence
1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	5.64	52	GLTSLDRYKGR
2	PsbO	5.85	33	LTYDEIQSK
				DGIDYAAVTVQLPGG
				ERVPFLFTIK
				GGSTGYDNAVALPA GGR
3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	5.64	25	DTDILAAFR
4	Trypsin inhibitor	5.97	20.1	VTGQSCPEIVVQR
				GSDDDGIPVIFSNADG K
				LDDYDPSTGR
				ATGQSCPEIVVQR

Protein profiles: Analysis of protein profiles by eletrophoretic techniques has been used to study and understand biological and physiological mechanisms of plants under normal and most diverse stress conditions (Schiltz *et al.* 2004). Plants develop sophisticated response mechanisms for defense and protection against several stress factors (Holley *et al.* 2003). The electrophoretic profile observed in the present study may be the result of seedling adaptation to adverse conditions generated by a biotic stress. According to Spreitzer and Salvucci (2002), the Rubisco form found in leaves of mutants and wild seedlings, known as form I, is constituted by large as well as small subunits and is present in most photosynthesizing organisms (Fig. 6). The alterations of cellular proteins at the leaf level can function as a defense mechanism (Figs. 7, 8). Thus, in many cases response mechanisms are based in the expression of defense genes and alterations in cell proteins (Schiltz *et al.* 2004).

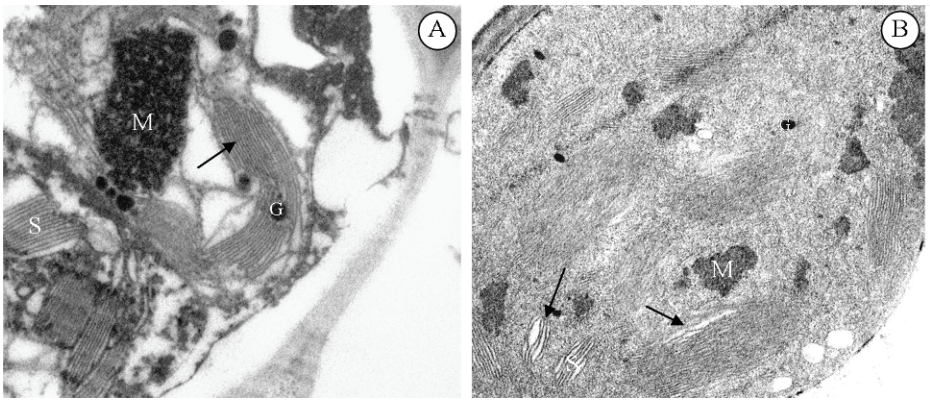

Fig. 9. Transmission electron micrography of mature leaf chloroplasts (*arrows*) from wild-type (*A*) and mutant (*B*) seedlings, fixed in glutaraldehyde and OsO_4, embedded in plastic resin, and thin-sectioned with an ultramicrotome. Magnification 22,000 ×. S – stroma; M – mucilage; G – starch grains.

The sequencing of proteins by mass spectrometry analysis of group 3 from the 2D gel (Fig. 7), revealed the existence of two peptides in the mutant and two in the wild type, whose concentration was higher in the wild type, with 100% similarity with PsbO, a protein of PSII stabilizing its oxygen evolving Mn cluster (Table 7, Murakami *et al.* 2005). The concentration of PsbO is a critical limiting factor for photosynthesis and plant growth and may also limit the concentration of other proteins such as PsbA and PsbP (Murakami *et al.* 2005). PsbA provides ligands for cofactors and inorganic ions that catalyze the oxidation of H_2O, which make the transfer of electrons to PSII (Nelson and Yocum 2006). Furthermore, Liu *et al.* (2007) studies with artificial mutants of *Arabidopsis thaliana* revealed that this species contains two genes that express two PsbO proteins (PsbO-1 and PsbO-2). These authors showed that the PsbOs are related to the efficiency of the photochemical stage of PSII and to the D1 protein of the PSII reaction center. Mutant PsbO-1 deficient plants showed significant damage of the PSII photochemical step (Liu *et al.* 2007). The same may have occurred with the natural mutant of *T. cacao*, causing an obstruction in photosynthetic electron transport, therefore, preventing the plants to photosynthesize. Nevertheless, we cannot exclude that the *Luteus-Pa* mutation also affects the level of other crucial PSII subunits and the decrease in the PsbO content in the mutant leaves represents just an indirect influence of their depletion.

Sugars and starch: According to Almeida *et al.* (1998), seedlings bearing the lethal factor do not photosynthesize and are necessarily maintained by its cotyledonary reserves showing a negative CO_2 balance in relation to wild-type seedlings. This fact was confirmed in the present work, which shows a tendency for lower TSS and starch concentrations in all vegetative organs of mutant seedlings at 15 DAE (Table 5). This also shows the existence of high demands for cotyledonary metabolites. Huber (1983) reported that more than 50% of the carbon fixed by photosynthesis can be used in the formation of starch and sugars depending upon species, environment, nutritional status and developmental stage. This partitioning is important for plant growth, as well as sucrose formation, sugar transport and determination of carbon export by photosynthesizing leaves (Huber 1983).

Chloroplasts: Contrary to chloroplasts in mesophyll cells of fully expanded green leaves in wild-type seedlings, the leaf plastids of the mutant presented a disorganized stroma lamellae with spaces between thylakoids membranes (Fig. 9), that could have contributed to the inefficiency of their photosynthetic apparatus. In a study accomplished by Whatley (1992), also in fully expanded leaves of *T. cacao*, he observed that each chloroplast contained (*1*) 5–7 lamellar bands, (*2*) most grana contained 5–7 compartments, but some stacks had up to 20 compartments and (*3*) starch grains, similar to chloroplasts of the wild type seedlings in the present study.

Pigments: The decrease in the concentrations of Chls *a*, *b* and total observed at 15 DAE in mutant seedlings when compared to the wild counterparts (Table 6) was similar to that previously observed by Almeida *et al.* (1998). The decrease in Chl concentration and leaf chlorosis, followed by visible necrosis in the mutant seedlings, indicate induced senescence by the presence of the *Luteus-Pa* factor (Almeida *et al.* 1998). The degradation and synthesis processes of Chl can be influenced by endogenous and exogenous factors (Ortensteiner 2006). Chl molecules are sensitive to pH, enzymes, temperature, light and oxygen, which can exert more or less influence in their degradation (Bohn and Walczyk 2004). The decrease in carotenoids content (Table 6) in mutant seedlings can be justified by the occurrence of injuries in PSII, since carotenoids, as well as Chls are part of PSI and PSII and synthesis as well as degradation of these pigments are normally associated to photosynthetic efficiency (Baker 2008). Some of the most important functions of carotenoids are the dissipation of excess energy from the excited Chls and the elimination of reactivate oxygen species (Lawlor 2001), whose formation is prompted under conditions of oxidative stress (Schützendübel and Polle 2002), as shown in the

present study.

Its concluded that: (*1*) the seedlings resulting from the reciprocal crosses of Pa 30 and Pa 169 did not show differences in the segregation proportion of the lethal gene *Luteus-Pa*, therefore, it is possible to affirm that the inheritance of the gene is nuclear; (*2*) the leaves of mutants seedlings of both crosses did not photosynthesize, just respired; (*3*) there were damages in the antenna complex and, or reaction centers of PSII, impairing transfer of excitation energy; (*4*) variations in growth parameters and consequent death of mutant seedlings was induced by the exhaustion of cotiledonary reserves, inoperance of the photosynthetic apparatus and oxidative stress, (*5*) the mutation induces alteration in the quantity of protein in few spots and chloroplast morphology.

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